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Ecology and Molecular Genetic Studies of Marine Bacteria

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Project Summary

The major objective of the project is to study the ecology, molecular genetics, and evolutionary relationships of marine and estuarine bacteria as a means of understanding their survival, growth, and distribution in the environment. To achieve this objective, research has been conducted in four basic areas: 1) detection and survival of bacteria in aquatic environments; 2) selected aspects of the microbial ecology of the deep ocean and hydrothermal vent systems; 3) molecular characterization of ecologically important genes; and 4) systematic analyses of members of the genus Vibrio and related genera, which are commonly isolated from the estuarine and marine environment.

Results to Date

Survival and detection of bacteria in aquatic environments. One of the major limitations to research in microbial ecology has been the inability to isolate and grow in culture the vast majority of bacteria which occur in nature. The occurrence of non-culturable bacteria has long been known (Bisset, 1952; Hoppe 1978), but the nature of the phenomenon has not been determined. Colwell et al. (1985) reported that some pathogenic bacteria lost the ability to grow on laboratory media after incubation in oligotrophic ocean water or microcosms for short periods of time (less than 1 day to 3 weeks), while cell numbers changed little by direct microscopic counts. The implication of these observations is that pathogens surviving in the environment may not be detected by standard methods. Grimes and Colwell (1986) investigated the survival of pathogenic Escherichia coli in membrane chambers submerged in semitropical ocean water, and reported that viable cells could be detected by the direct viable count (DVC) method (Kogure et al., 1979) long after they could be cultured on laboratory media. Introduction into a ligated rabbit ileal loop led to the recovery of cells which grew in culture and demonstrated plasmid profiles identical to those of the original inocula. Results of this study suggested that water-borne pathogens which elude detection in the laboratory may retain their pathogenicity, and may be "revived" to the culturable state by animal passage. Tamplin and Colwell (1986) evaluated the effect of salinity and organic concentration on the production of enterotoxin by Vibrio cholerae in microcosms.

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Results showed that at both high (10 g/l) and low (1 g/l) concentrations of organic nutrient, toxin production increased with salinity, in the range of 0 to 25 ppt. Thus, bacteria not only survive exposure to the marine environment, previously believed to lead to rapid die-off, but retain important properties, including potential pathogenity.

Much of the work with non-culturable bacteria requires direct counting methods which assay the total number of cells present in a sample. The DVC method (Kogure et al., 1979) has been widely used as a means of estimating metabolically active bacterial populations. DVC counts are generally much higher than counts obtained by plate counts on agar media, and lower than Acridine Orange Direct Counts (AODC) (Hobbie et al., 1977). Recently a strong correlation has been observed between DVC counts, heterotrophic activity in natural samples and metabolic activity by microautoradiography (Kogure, et al. 1987; Roszak and Colwell 1987a), suggesting that the DVC method provides a good estimate of the viable bacterial population and substantiates the existence of a viable but nonculturable state.

Roszak and Colwell (1987b) reviewed the literature on survival strategies of bacteria in aquatic environments and proposed a resting or "somnicell" stage for Gram-negative cells, analogous to spore-formation in some Gram-positive bacteria. Recently, we have investigated the possibility of a genetic basis for nonculturability by probing DNA isolated from Vibrio using probes prepared from cloned spo genes of Bacillus. Preliminary results show a significant level of homology between discrete fragments of the Vibrio genome and the spo probes in Southern blot analyses (Gobius et al. 1989, submitted). The function(s) of the relevant Vibrio genes in vivo is not yet known, but the genes have been cloned into Escherichia coli and are presently being sequenced. The cloned genes can also be used to generate specific mutations in the native host via single-crossover insertion mutation (Miller and Mekalanos, 1988) followed by assays for retention or loss of the non-culturable state.

The inability to culture the majority of bacterial cells in sea water has slowed progress in marine microbiology. During the past year we have circumvented cell culture by exploiting other characteristics of bacteria which may provide important information about the cell and how it interacts with the environment. Knight et al. (1988) studied bacteria within shark tissues by monitoring for the presence of ureolytic enzymes. By studying tissue homogenates, ureolytic bacteria were detected in shark liver, but not in blood, without the need for classical cell culture. Cell-surface antigens can also be used to detect or track bacteria in the environment. Extensive work has been done on the production of both poly- and monoclonal antibodies to environmentally important bacteria (Tartera et al., 1989). These antibodies are routinely used in conjunction with direct counting and DVC procedures to enumerate total and viable numbers of specific organisms within the total bacterial population in seawater.

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It is also possible to detect bacteria in a habitat by the presence of specific gene sequences. In fact, the ability of aquatic bacteria to transfer genetic information horizontally implies that important genetic traits may be propagated in the environment even after the demise of the original host (Grimes et al., 1986a). Therefore, tasks such as tracking genetically engineered microorganisms (GEMS) in the ocean should not be by cell detection alone, but should be supplemented by detection of the relevant genes. We have recently developed a procedure by which total cellular RNA and DNA, including plasmid DNA, can be isolated from relatively large volumes of water samples without the need for cell culture (Somerville et al., 1989). Nucleic acids purified by this method are suitable for targets of DNA/DNA or DNA/RNA probes, restriction digestion, and sub-cloning procedures, or templates for direct sequence determination. Isolation of ribosomal RNAs by this method make it possible to identify the bacterial component of an aquatic habitat without growing the cells, and the isolation of mRNA make it possible not only to detect species present, but the genes being expressed. Direct isolation of nucleic acids from environmental samples is complemented by molecular characterization of ecologically important genes (vide infra).

Detection of specific gene sequences in the environment by DNA probes may be complicated by the relatively small number of cells of the parent organism and large volume of water, with the result that the gene may be present in very low concentration. We have attempted to overcome these difficulties by developing a probe method which uses the sensitivity of DNA oligomers (approximately 20-30 bp) and adds a long tail of radiolabeled or otherwise tagged DNA to enhance detection (Somerville et al., 1988 Abstract). The method takes advantage of the potential of oligomer probes to act as polymerization primers and eliminates background signal by blocking free 3' hydroxyl residues with dideoxynucleotides. Preliminary results suggest that this method may enhance sensitivity by as much as two to three orders of magnitude over conventional oligomer probes.

Another aspect of monitoring bacteria in the environment is the detection of culturable organisms which occur in such low numbers that their presence may be missed by standard microbiological tests. This points to another weakness of standard bacteriological assays, and that is the small sample size which can be evaluated. Knight et. al. (1989) have attempted to avoid the limitations of sample size by concentrating biomass from relatively large samples of seawater onto Sterivex (Millipore) membrane filters. This type of filter serves as a convenient culture vessel to which selective or enrichment media can be added, allowing culturable cells which are present in very low numbers to grow to large cell numbers. These tests are not only more sensitive, but are rapid and simple, and will improve detection of indicator organisms in the environment (Elliot and Colwell, 1985).

Colwell et al. (1988) suggested that understanding the fate and effects of ecologically important microbes, in the ocean may require an integrated effort, including cultural techniques supplemented by gene probes and immunological methods, and supported by a detailed taxonomic structure.

Microbial ecology of the deep ocean and hydrothermal vent systems. One of the most exciting, yet least understood areas in aquatic microbiology is the ecology of the deep ocean. We have continued to study bacteria from deep sediments, and have investigated the microbial ecology of hot submarine vent systems. Culturing deep-sea bacteria requires that we mimic in situ conditions in the laboratory. Unfortunately, holding bacterial cultures under high pressure requires the use of cumbersome pressure vessels which are not easily adapted to cell culture, and incubation at temperatures characteristic of the deep ocean results in very long generation times. We have made improvements in our ability to grow the obligately barophilic bacterium BNL-1 (Deming et al., 1984) by constructing four large pressure vessels, which can hold a full liter of culture under in situ pressure and temperature. We have also constructed a specialized apparatus which can rock two of the vessels during incubation. Results indicate that use of rocking incubation increases cell yield by approximately 10-fold over a 10 day incubation period. Because of these improvements in culture technique, we have been able to produce sufficient biomass of BNL-1 to yield chromosomal DNA suitable for establishment of a gene library. We have approximately 3000 presumptive clones which can be screened for known gene sequences or enzymatic activities. The bank has been screened for the production of enzymes that hydrolyze chitin oligomers.

Barophilic bacteria are those which grow more rapidly (facultative barophiles) or exclusively (obligate barophiles) at pressures greater than 1 atm. While a number of barophilic bacteria have been isolated (Deming et al., 1984; Deming, 1985; Deming et al., 1988; Morita, 1980; Yayanos et al., 1981), they remain poorly characterized phenotypically. This may be due to technical problems associated with limited space in pressure vessels, the long doubling time of many psychrophilic barophiles (4 to 12 h), or the lack of simple assays for enzyme activity under pressure. We have developed a colorimetric method for detection of 19 constitutively expressed enzymes under in situ conditions of pressure and temperature using a simple modification of the commercially available API ZYME enzyme assay kits (Straube et al., 1989a). Eleven strains of barophilic bacteria, including an obligate barophile, were examined. With the API ZYME color chart, the enzyme reactions could be divided into negative (grade 0), weak (grade 1), and positive (grades 2 through 5). Of the 10 facultatively barophilic isolates, 9 exhibited a change of phenotype in at least 1 enzyme when tested at 1 atm vs in situ pressure. The assay is simple, relatively rapid, and allows for direct determination of enzyme activity

under high pressure and low temperature conditions.

The Sterivex method for recovery of nucleic acids from aquatic samples (Somerville et al., 1989) has also proven useful in characterizing bacteria from the deep ocean. Preliminary tests of the method were conducted in 1987 on samples collected in and around hydrothermal vents located on the Endeavor ridge. In these experiments, it was found that enzymatic lysis did not yield detectable concentrations of DNA, particularly from relatively pure smoker fluid samples. However, when the crude enzymatic lysates were subjected to French press lysis and partially purified, DNA concentrations as high as 16 ng/ml of smoker fluid were recovered. The data suggest the presence of cellular structures in hot vent fluids which are recalcitrant to enzymatic lysis. We have succeeded in cloning small fragments of this DNA into pUC18 and are preparing to sequence the clones in an attempt to characterize their source.

In a second study, as part of an interdisciplinary survey of hydrothermal vents on the Endeavor segment of the Juan de Fuca Ridge, the submersible ALVIN was used to collect 60 fluid samples in titanium syringes from 17 different smokers and their environs. Nucleic acids were extracted from the samples and concentrations of partially purified DNA were determined spectrofluorometrically employing Hoechst 33258 (Paul and Myers, 1982). Correlations between DNA concentration and the ratio of seawater and hydrothermal fluid in the field samples, determined by geomarkers (pH, Mg and Si content, and temperature), support the following hypotheses: (i) smoker environments are heterogeneous, with respect to history, location, chemistry, and microbiology; and (ii) a unique microbial population exists in the fluids of some smokers and those fluids (and contained biota) disperse rapidly into surrounding seawater and rise to create a buoyant plume, which then supports the establishment of a second microbial population. Detailed sampling at one smoker revealed DNA concentration of 0.86-1.32 ng/ml in superheated (357°C) waters containing 85% hydrothermal fluid, 0.09-0.36 ng DNA/ml in ambient seawater (containing 0-1% hydrothermal fluid) near the smoker throat, and 1.13-1.58 ng DNA/ml in the buoyant plume 20-100 m above the smoker. Given these measurements, the presence of particulate DNA in the emergent superheated smoker fluid cannot be attributed to entrainment of ambient seawater into the hydrothermal fluid, either naturally through the porous sulfide walls or as a sampling artifact (Straube, et al., 1989b). Much of the variability in DNA recovery observed in the current research supported by this project can be ascribed to geographical location within the Endeavor vent field. These differences may be a result of factors such as smoker age, flow rate, and geochemistry, which in turn lead to heterogeneity in the biotic content of the fluids. Therefore, the inability to culture bacteria from some hot vents should not be used to conclude that such life does not or can not exist in all vents.

Molecular characterization of ecologically important genes. Microbial ecology is currently progressing both in the isolation and characterization of microbiota and in the isolation and characterization of biomolecules from the environment. The characterization of environmentally important genes in the laboratory provides information useful in preparing gene probes for detection of specific bacteria or nucleic acids as indicators of environmental conditions.

Environmentally important genes were studied by describing a 34 Mdal plasmid from an estuarine isolate, Flavobacterium sp. SB23, which coded for the degradation of phenanthrene (Okpokwasili et al., 1986). Plasmid DNA from this isolate can be used to probe for similar genes in other environments, and may be useful in identifying areas which have been exposed to hydrocarbon pollution. Probes can be made against colony blots or against DNA purified directly from the environment. The latter technique is advantageous in that it minimizes non-specific probe/target interactions, and obviates problems posed by nonculturable bacteria (Somerville et al., 1989).

Chitin represents a major carbon and nitrogen reserve in the aquatic environment. As such, organisms which possess the capability to hydrolyze chitin play an important role in nutrient cycling, not only among procaryotes, but throughout the entire food web. In 1986, we reported the cloning and initial characterization of chitinolytic determinants from Vibrio vulnificus (Wortman et al., 1986). At that time we believed the cloned DNA coded for two or three proteins. The hydrolysis of chitin polymers is known to proceed via two enzymes: (i) chitinase, which hydrolyses high molecular weight chitin predominantly to chitobiose; and (ii) chitobiase, which hydrolyses chitobiose to the monomer sugar (Jeuniuax, 1966). Our clones also complemented a lacY deficient strain of Escherichia coli, leading us to believe that a permease might also be encoded. Since that initial report, we have completed the sequence analysis of the cloned DNA and have expressed the insert in an in vitro transcription and translation system. Both the sequence analysis and expression predict a single, 94 kDa product from the cloned DNA (Figure 1; Somerville and Colwell, manuscript in preparation). This single enzyme appears to have hexosaminidase activity, hydrolyzing chitin oligomers by sequential removal of individual NAG residues from the non-reducing end of the molecule. In the native host, V. vulnificus, chitin degradation may be due solely to the production of the hexosaminidase, or may be a function of a suite of enzymes including chitinase and chitobiase. To examine this question we have undertaken the production of mutants at the hexosaminidase locus in V. vulnificus. This is done by cloning a large fragment of the gene into vector pGP704 (Miller and Mekalanos, 1988) and mobilizing the plasmid into the target organism. pGP704 is replicated in the parental host, E. coli SM10, due to the presence of the pir polymerase gene on a lysogenized lambda construct. When mated with V. vulnificus, SM10 is capable of moving the plasmid into

the recipient via conjugation, but once transferred, the plasmid cannot replicate due to the lack of the required polymerase in V. vulnificus. The plasmid not only carries a portion of the gene of interest, but also has been engineered to carry an ampicillin resistance marker. Media containing ampicillin are used to detect transconjugants that have retained the marker by recombination into the chromosome. Since the cloned gene represents a major region of homology with the target chromosome, recombination is most likely to occur within the gene, leading to an insertional mutation at this locus. To date we have isolated 16 V. vulnificus transconjugants, and are testing each for the ability to hydrolyse colloidal chitin. Each strain will also be assayed for recombination at the hexosaminidase locus by Southern blot analyses using the originally cloned gene as a probe.

The potential for two or more different chitinolytic schemes in aquatic bacteria, which we observed during our study of Vibrio vulnificus, has led us to look more carefully at chitin hydrolysis in environmental isolates and the methods used to detect chitinolytic microbes. Bacteria isolated from Chesapeake Bay sediments were screened for chitin hydrolysis using methylumbelliferyl-N-acetylglucosamine (MU-NAG), methylumbelliferyl-N,N'-diacetylchitobioside (MU-chitobiose), methylumbelliferyl-N,N',N''-triacetylchitotrioside (MU-chitotriose), and chitin overlay agar. Bacteria inoculated into micro-titer wells containing LB broth plus MU-substrate were monitored for production of fluorescent hydrolysis products for up to 48 hours. Isolates plated on colloidal chitin overlay agar were monitored for zones of clearing for up to 45 days. Patterns of hydrolysis varied widely, both with respect to the substrates hydrolyzed and the time elapsed before a positive response. Lack of hydrolysis of the MU-substrates correctly predicted lack of chitin clearing in most cases. Bacteria which produced zones of clearing on chitin agar usually hydrolyzed the MU-substrates after 48 hours incubation, but the opposite was not true. None of the MU-substrates accurately predicted clearing of chitin agar. The data suggest that MU-substrates, particularly MU-NAG, should be used with considerable caution as substitutes for chitin agar in determining the ability of bacterial isolates to digest native chitin (Somerville et al., 1989).

Systematic analyses of members of the genus Vibrio and related genera. Microbial ecology requires that bacteria or biomolecules isolated from the environment be identified quickly in order to reveal timely information about the habitat in question. Experience has shown, however, that limited phenetic analyses for the classification of bacterial isolates may lead to assigning them to improper taxa. Therefore the boundaries of the family Vibrionaceae and related taxa have been delineated phylogenetically. The result of defining taxa along evolutionary lines will be the ability to choose key phenetic characters which will be phylogenetically rooted and, therefore, reliable tools for bacterial classification. Table 1 lists strains of Vibrio

and related genera which have been characterized in this laboratory by rRNA sequence analysis, DNA/DNA homology, and/or numerical taxonomy. We are in the process of comparing phylogenies predicted by the three methods determine the best method of a defining a natural classification of these organisms.

In recent years "molecular clocks" have been used to define evolutionary relationships among bacteria. The ideal clock is an essential macromolecule which is common to all isolates and has both highly conserved and variable domains. The commonality of the clock indicates that it has been inherited from a common ancestor, and the conserved and variable regions allow for measures of change over long and relatively short periods of evolutionary time, respectively. The nucleic acid constituents of the ribosome, ribosomal RNAs (rRNAs), satisfy the conditions of a molecular clock. By determining the base sequence of rRNAs it is possible to make direct comparisons between organisms and groups of organisms which will reveal the extent of their divergence. Several new techniques for the isolation of 5S rRNA, and the analysis of nucleotide sequences have been developed in this laboratory. They include the isolation and purification of RNA by High Performance Liquid Chromatography (HPLC; MacDonell et al., 1986c), analyses of 5S rRNA secondary structure (MacDonell and Colwell, 1985a), novel approaches to sequence analysis (MacDonell et al., 1988), as well as computer programs for comparative sequence analysis (MacDonell and Colwell, 1984b), and the computation of free energy for a proposed secondary structure in an RNA molecule (MacDonell and Colwell, 1984a).

We are continuing to investigate the systematics of marine and estuarine bacteria by means of similarity analyses based on 5S rRNA sequences. We have compiled the complete 5S rRNA sequences of 64 members of the genus Vibrio and related genera and are currently working on 8 additional sequences (Table 1). Of particular interest are relationships between the aeromonads, alteromonads, photobacteria and vibrios, all of which are taxa commonly isolated from seawater, marine sediments, and marine biota. Sequences collected to date have allowed construction of a phylogeny for these bacteria (MacDonell et al., 1986b), and insight into the variability of mutation rates in eubacterial 5S rRNA (MacDonell et al., 1986a). We have nearly completed 5S rRNA sequences for all the type strains of the genus Aeromonas and have found them to possess a unique and characteristic base sequence at the helix and loop in the secondary structure. The genus Aeromonas, in fact, is sufficiently phylogenetically deep to warrant elevation to the family level (Colwell et al., 1986). Completion of the Aeromonas data base will allow us to determine phylogenetic relationships between aeromonads and vibrios. The systematics of the alteromonads is also being clarified by 5S rRNA sequencing. The sequence analysis of Alteromonas hanedai has allowed us to recommend that this species be reassigned to the genus Shewanella (Coyne et al., submitted).

An extensive collection of sequences is required before phylogenetically relevant information can be obtained. The 5S

rRNA sequences of 50 strains were used to generate the dendrogram shown in Figure 2. Three major clusters form at a similarity value of 0.9. These clusters indicate distinct phylogenies for the genera Aeromonas, Shewanella and Vibrio. The Vibrio cluster comprises 4 groups of strains which cluster at a similarity value of 0.93 or greater. The taxonomic position of these groups is the subject of continuing research.

Another method of determining relatedness among a group of organisms is to measure the extent of homology between pairs of genomes by DNA/DNA hybridization. The method is based upon the assumption that organisms which share a very recent common ancestor will have a large degree of homology between their genomes. As the time since divergence increases, the extent of change from the ancestral genome should also increase, leading to lower homology between pairs. We have recently completed the determination of DNA/DNA homologies for all pairs and reciprocal pairs of 31 type strains of Vibrio and Photobacterium. In this method chromosomal DNA is isolated from both members of a pair, and the DNA from one member of the pair is labeled by nick-translation and sheared by sonication. The shearing of the probe DNA reduces the size of the labeled strands, thus minimizing non-specific tailing of heteroduplexes. Target and probe DNAs are denatured, mixed, and allowed to anneal. Duplexes are then separated from single strands by hydroxyapatite chromatography, and the amount of radioactivity contained in heteroduplexes is measured by scintillation counting. Identical pairs are used to define complete homology under the conditions of the assay, and the homology of a heterologous pair is expressed as a relative binding ratio (RBR). RBRs determined by this method have been used to construct the dendrogram shown in Figure 3, based on maximum RBR values for reciprocal pairs. Using this type of analysis, 3 major clusters form at the 0.19 level which appear to have phylogenetic significance. The cluster which contains the majority of the vibrios includes two distinct groups which form at a similarity value of 0.2 (see Table 2).

Determinative classification schemes, which are based on a small number of phenetic characteristics, require a priori assignment of significance to certain phenotypes which are used to differentiate bacterial taxa. In some cases, however, these determinative characters do not reflect phylogenetic relationships. One method of avoiding the pitfalls of determinative schemes is to maximize the number of phenetic characters assayed, and to give equal statistical weight to every character in the final analysis. This approach, numerical taxonomy (NT), entails assaying (>100) phenetic characters from a large number (sometimes hundreds) of bacterial isolates. Specialized sorting algorithms are then used to produce dendrograms based on the number of shared characters between individuals and clusters of organisms. Reference organisms are included in the analyses to facilitate identification of the clusters. Figure 4 shows a phenogram generated by numerical taxonomy of vibrios and related environmental and clinical

isolates, based on Euclidean distance coefficient and unweighted-pair-group arithmetic sorting, with distances transformed into percentages of similarity (West et al., 1986). The authors defined 23 phena which contained 400 of 443 isolates tested. This dendrogram shows 3 major clusters which form at approximately 73% similarity. Several smaller groups are defined at $\geq$ 83% similarity (see Table 2).

A detailed treatment of the information in Figures 2, 3 and 4 is given in Table 2. Strains listed under rRNA sequence analysis, DNA/DNA homology and numerical taxonomy are, from top to bottom, exactly as they appear in Figures 2, 3 and 4 respectively. The clusters and groups listed in Table 2 are presented to compare results of procedures described above, and are not intended to represent coherent taxa. The value of rRNA sequence analyses, DNA/DNA homologies, and NT studies in a polyphasic approach to taxonomy is the subject of continuing research.

Ecology and molecular genetics studies of marine bacteria. While our research progresses in four distinct areas, each area contributes to a central, the ecology of bacteria in aquatic environments. Detailed analyses of environmentally important genes provide us with the means to detect specific bacteria in the environment without the need for cell culture, but they also provide us with information regarding the evolution of these genes and the host organisms. The seemingly esoteric study of bacterial evolution, in turn, will provide phylogenetically meaningful characters which can be used for rapid and efficient identification of marine bacteria which can be cultured from the environment. As the ability to characterize and monitor bacteria in the ocean is improved for extreme environments such as the deep ocean and thermal vents, even the non-culturable forms will be able to be characterized.

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